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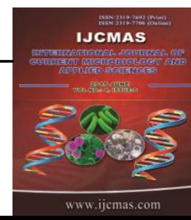

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Original Research Article

Analysis of IR, NMR and invitro antibacterial Potency of *Pistacia integerrima* against 6 Clinically Isolated Multidrug Resistant Bacteria

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Keywords

Pistacia integerrima, Gram-positive bacteria, Gram-negative bacteria, Multidrug resistant bacteria, Minimum inhibitory concentration, Antibacterial activity, Phytochemical constituents, GC IR. NMR.

A B S T R A C T

To investigate the antibacterial activity, isolation and identification of pure compound using extraction procedure with five solvents, hexane, chloroform, ethyl acetate, methanol and water to validate medicinal uses of *Pistacia integerrima* (*P.integerrima*) in controlling infections; and to qualitatively estimate phytochemical constituents of galls-extracts of the plant.The antibacterial activity of galls-extracts was evaluated by the agar-well diffusion method against clinically isolated 6 Gram-positive and -negative multidrug resistant (MDR) pathogenic bacteria *in vitro*. Values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of galls-extracts against each bacterium were obtained in a 96-well micro-titre plate, by broth dilution micro-titre plate technique. To isolate pure compounds with a hope that they might be active against MDR bacteria cell, the chloroform extract of *Pistacia integerrima* showing maximum activity were subjected to TLC and finally pure compounds were subjected to IR and NMR analysis for structural elucidation.The presence of tannins, flavonoids, sterols, alkaloids, anthraquinone and coumarinsin different galls extracts was established. Pathogenic bacteria used were, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* and vancomycin resistant *S. aureus* along with standard bacterial strains. These MDR bacteria had been recorded to have significant inhibitions by galls extracts, obtained by extraction procedure with five solvents. The chloroform extract against *Staphylococcus aureus* had the highest inhibition zone-size (14 mm). Cefotaxime 30 μg/disc was the positive/reference control and the diluting solvent, 10% dimethyl sulphoxide was the negative control. Recorded MIC values of different extracts ranged between 0.48 and 13.20 mg/mL, and MBC values were 2.58 to 30.00 mg/mL, for these bacteria. Galls-extracts with water and chloroform had shown significant antibacterial activity against bacteria. On the basis of spectroscopic data found compound 1 was proposed to be a β-sitosterol. This study suggests that *Pistacia integerrima* galls-extract can be could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine.

Introduction

Presently in developing countries, synthetic drugs are not only expensive and inadequate for the treatment of disease but are often adulterated and show side effects (Shariff ZU. 2001). So there is a need to search for plants of medicinal value. Throughout history, plants have provided human with an essential source of medicine (Foster. 1999).The World Health Organization estimated that about an 80% population of developing countries relies on traditional medicines, mostly plant drugs, for their primary health care needs(WHO 2008, and Loeraa JA, *et al*., 2007). Particularly in rural India, uses of raw plant products as well as some concoction of plant products in Ayurvedic medicines are sought after to a great proportion, because of cheap availability, and in urban areas too those are increasingly popular for cultural nuances that exist(De Silva T, *et al*., 2009).In ethno-botanical literature of India, several hundreds of plants are known to have the potential to treat many diseases and one of those popular ones *Pistacia integerrima* (*P.integerrima*).

Pistacia integerrima belonging to family Anacardiaceaeis a native to Asia widely distributed in south Alpine Himalayas, Pakistan, Afghanistan. Growing at an altitude of 800-1900 m (Pant S, *et al*., 2010). It is an important medicinal plant and is used as anti-inflammatory, antidiabetic agent, a blood purifier, a remedy for gastrointestinal disorders and as a expectorant. In India it is used as an herbal remedy for aliments such as cough, asthma, fever, vomiting and diarrhea (Pant S, *et al*., 2010 and Uddin G, *et al*., 2012).it is hepatoprotective (Uddin G, *et al*., 2011). Galls in combination of other drugs are also used against snake bite and scorpion sting (Ahmad S, *et al*., 2010); and Galls possess antimicrobial property (Uddin G, *et al*., 2012). Possess antiviral property (Mutail C, *et al*., DOI;4172).Stem resin is used in wounds healing (Hussain F, *et al*., 2007).Leaves are used as fodder for cattle (Jan S, *et al*., 2008).

Infections with both Gram-positive (GP) and Gram-negative (GN) bacteria have clinically become intractable, slowly, due to the emergence of multidrug resistant (MDR) strains. Among GP pathogens, strains of *Staphylococcus aureus* (*S. aureus*), methicillin resistant *S. aureus* (MRSA) and vancomycin resistant *S. aureus* (VRSA), strains of *Enterococcus* sp. are noteworthy (Dubey D,*et al*., 2012).Moreover, GN bacteria, *Acinetobacter* sp., *Klebsiella pneumoniae* (*K. pneumoniae*), *Citrobacter freundii* (*C. freundii*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are commonly found as pathogens of urinary tract; while *E. coli, K. pneumoniae,* are pathogens of gastrointestinal tract. Presently, these pathogens are too MDR, recorded in several reports (Dubey, *et al*., 2012).

The resistance of pathogenic bacteria to antibiotics is of high clinical concern. Rather the concept of the control of drug resistance is a matter of clairvoyance for dovetailed antimicrobials today. A suitable epitome is the superbug, multidrug resistant (MDR) *S. aureus* in the human health domain worldwide, as its different strains or rather incarnations have generated β-lactamase activities in degrading all sorts of penicillin derived antibiotics, in addition to resistance to other groups/generations of antibiotics (Clarke CR. 2006).Multidrug resistance of *Staphylococcus, Pseudomonas, Escherichia* and a few more pathogenic bacteria to a wide range of antibiotics has been reported to have been due to non-prudent uses of same antibiotics against infections of food- and pet-animals worldwide(Middleton J,*et al*., 2005).

Including man(Maple PAC, *et al*., 1989). MDR-MRSA strains carry resistance markers for other antibiotics and instances of resistance up to 23 antibiotics in some strains have been reported (Middleton J,*et al*., 2005).

The emergence of VRSA is of further concern. Today, the management of the consortium of MDR strains of both GN and GP pathogens has become increasingly difficult because of the β-lactamase production by *Staphylococcus, Bacillus, Pseudomonas, Proteus, Klebsiella, Neisseria, Salmonella, Haemophilus* and a few more pathogens(Rath S,*et al*., 2012);and pandrug resistance (PDR, resistance of bacteria to all antibiotics in present use) to different classes of antibiotics in GN ones (Falagas ME, *et al*., 1989).

Meek appreciation of failures in the control of MDR strains would be inhuman, which generates the impetus on a systematic global search for new drugs from natural resources like plants, worldwide (Davidovich C, *et al*., 2008 and Dubey D, *et al*., 2012); chemicals from plants could be chosen for the control in a future crusade against MDR pathogens. Moreover, accumulated ethnomedicinal reports of different countries lend themselves well to the basic information needed for further work on drug-targeting against MDR pathogens (Dubey D, *et al*., 2012).

In the present study, crude galls extracts of *Pistacia integerrima* with 5 solvents, hexane, chloroform, ethyl acetate, methanol and distilled water were used to monitor antibacterial property against 6 clinically isolated MDR bacterial strains and isolated the bioactive compound-1 from *Pistacia integerrima* characterize by TLC, IR and NMR spectral analysis.

Materials and Method

Preparation of Plant Extract

The air-dried powdered galls material (in 40 g lots) of *Pistacia integerrima* was extracted with 400 mL volumes of solvents, hexane, chloroform, ethyl acetate, methanol and distilled water. By using soxhlet apparatus for solvent extraction done in a lot of 40 g of powder-mass was placed in the extractor and a volume of 400 mL of a solvent was used during 24 h of soxhletion, till colourless extracts precipitated in the extractor. After filtration, each extract was concentrated by the rotary evaporator.

The resultant sticky-mass was dried in a desiccator; the solid mass was stored in a suitable volume of 10% dimethyl sulphoxide (DMSO) with a drop of Tween-80. The solid extract was dissolved in a required volume of 10% DMSO and a drop of Tween-80 for a final concentration of 30 mg/mL. The stock concentration of each extract was maintained at 30 mg/mL, for further use.

Phytochemical Screening

Extracts of galls of *Pistacia integerrima* using hexane, chloroform, ethyl acetate, methanol and distilled water were subjected to various chemical tests in order to determine the secondary plant constituents:

Test for the Presence of Anthraquinones

An aliquot of 0.5 mL of the extract was shaken with 10 mLof benzene, filtered and an aliquot 5 mL of 10% ammonia solution was added to the filtrate and the mixture was shaken, the presence of a pink, red or violet colour in the ammoniac (lower) phase indicated the presence of anthraquinones (Sofowara A. 1993).

Test for Saponins

An aliquot of 0.5 mL of an extract was dissolved in an aliquot of 10 mL of distilled water in a test-tube was shaken vigorously for 30 s and then allowed to stand for 45 min. The appearance of a frothing, which persists on warming indicated the presence of saponins(Brain KR, *etal*., 1975).

Test for Flavonoids

To a portion of the dissolved extract, a few drops of 10% ferric chloride solution were added. A green or blue colour indicated the presence of flavonoids (Brain KR, *etal*., 1975).

Test for Steroids/Terpenes

A lot of 500 mg of the extract from the rotary evaporator was dissolved in an aliquot of 2 mL of acetic anhydride and cooled at 0 to 4°C, to which a few drops of 12 N sulphuricacid were carefully added. A colour change from violet to blue-green indicated the presence of a steroidal nucleus (Sofowara A. 1993).

Test for Tannins

A fraction of 0.5 g of the extract was dissolved in 5 mL of water followed by a few drops of 10% ferric chloride. A blue black, green, or blue-green precipitate would indicate the presence of tannins (Brain KR, *etal*., 1975).

Test for Alkaloids

A lot of 0.5 g of ethanol extract (from rotary evaporator) was stirred with an aliquot of 5 mL of 1% HCl on a steam bath and filtrated; to an aliquot of 1 mL of the filtrate, a few drops of Mayer's reagent was added, and to another aliquot of 1 mL of the filtrate, a few drops of Dragendorff's reagent were added. Turbidity or precipitation in tubes due to either of these reagents indicated the presence of alkaloids in the extract (Brain KR, *etal*., 1975).

Test for Coumarins

Moistened plant extract (0.5g) was taken in a small test tube and covered it with filter paper moistened with 1N NaOH. The test tube was placed for few minutes in boiling water. Then filter paper was removed and examined in UV light for yellow florescence to indicate the presence of coumarins(Brain KR, *etal*., 1975).

Isolation and Identification of Pathogenic Bacteria

From (Kanchipuram Dist.) hospitalized patients of wards and cabins of Hospital, 6 bacterial strains (Three GP species, *S. aureus*, MRSA, VRSA.; and Three GN bacteria, *K. pneumoniae, E. coli and P. aeruginosa,*) were isolated. All these 6 strains were identified by standard biochemical tests and were maintained as axenic cultures in suitable media, as described previously (Dubey D,*et al*., 2012 and Rath S, *et al*., 2012).

Different clinical samples (Pus, Swabs, Urine, and Body Fluids) were collected from patients of wards, cabins, intensive care unit, neonatal care unit in the hospital, and were used for the growth of bacteria in nutrient agar,

MacConkey agar, blood agar, eosin methylene blue (EMB) agar, and xylose lysine deoxycholate (XLD) agar. Microbial type culture collection (MTCC) strain of each bacterium was used as the reference control during identification (see Table 1).

Antibiotic Sensitivity Pattern

All bacterial strains were subjected to antibiotic sensitivity test by Kirby-Bauer's method, using a 6 mm thick Mueller-Hinton agar medium, as described previously(Perez C, *et al*., 1990);and results were determined basing upon the standard guidelines(Clinical Laboratory Standard Institute Performance standard for antimicrobial susceptibility testing 2011). For the control, ciprofloxacin 30 µg/disc was used and it was sensitive to all test bacteria and its inhibition zone range was 16-19 mm.

Antibacterial Activity Tests

Antibacterial activity tests were performed both by agar well diffusion and disc diffusion methods. For the agar well diffusion method (Rath S, *et al*., 2012).bacterial lawn was prepared as described previously (Perez C, *et al*., 1990). but the agar was 6 mm thick. Wells (6 mm depth) were punched in 30 min old agar lawn and each well was based by 50 µL molten MHA medium. Further, wells were filled with 100 µL aliquots of 30 mg/mL solvent-extracts of *Pistacia integerrima* (diluted from the original stock by 10% DMSO to 30 mg plant extract/mL). Plates were incubated at 37°C for 24 h. Antibacterial activities were evaluated by measuring the diameter of zone of inhibition. It was confirmed that 10% DMSO had no inhibitory effect on the bacterium. Gentamicin 40 µg/mL was taken as the reference-control.

Antibacterial Activity and Determination of MIC and MBC

Antibacterial activities of plant-extracts were recorded by the agar-well diffusion method, as described previously (Rath S, *et al*., 2012 and Perez C, *et al*., 1990). The details of methods of determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were described previously (Sahu MC, *et al*., 2012).

Isolation of Phytochemical Compound

Chloroform fraction were selected on the basis of its antibacterial activity. Silica gel was used for column chromatography. Samples were loaded after adsorption on silica gel by making a uniform and even layer. Mobile phases starting from hexane to methanol in different ratios step by step were used. Different fractions obtained. All collected fractions were subjected to Thin Layer Chromatography (TLC). Solvent system used for TLC was chloroform and ethylacetate in the ratio of 50:50, 75:25, 40:60 but still minor impurities were observed. Further purification was carried out by preparative TLC. For mobile phase, chloroform and ethyl acetate were used in the ratio of 8:2. TLC plates were visualised under UV light at 254nm. A very fine single line was observed. Boundary lines were drawn and plates were cut from those boundaries to separate that part of TLC containing compound. Strips of TLC plates were cut in small parts, added in 250ml conical flask and first washed and ethanol successively 3-4 times to completely remove compound from the plate. Resultant sample were filtered and solvent was allowed to evaporate on rotary evaporator. Finally compound was confirmed for its purity and spectroscopic data were taken (Ahmad NS, *et al*., 2010).

The melting points were determined on a Perfit apparatus and are uncorrected. The IR spectra were recorded in KBr pellet on Win FT-IR S 135 Instrument (Bio-rad.USA).^{1}H (300MHz) and ^{13}C (75MHz) spectra were recorded by Brukerspectro- spin NMR

instrument in $CDCL_3$using TMS as internal standard. EIMS were scanned at 70 eV on jeol D-300 instrument (Jeol USA) (Ahmad S, *et al*., 2010).

Results and Discussion

Isolation and Biochemical Identification of Bacteria

Specific colony morphology of each pathogen was noted, for which a corresponding MTCC strain was used, parallely (Table 1). For example, After growth, a single colony was subjected to Gram-staining and basing upon it, other biochemical tests were performed for identification *E. coli* was negative for oxidase, Voges-Proskauer, citrate and urease tests; it was positive for catalase, indole, and methyl red, triple sugar iron and nitrate reduction tests. (Table 2).Similarly, the rest bacteria were typified. Three GPs, *S. aureus*, MRSA, VRSA. And three GNs, *E. coli, K. pneumonia.,* and *P. aeruginosa*, were isolated.

Phytochemical Analyses

From phytochemical analyses, it was ascertained that alkaloids were present in galls-extracts, obtained with ethyl acetate, methanol and water. flavonoids were present in extracts, obtained with hexane, chloroform, ethyl acetate and water. Tannins obtained with chloroform, ethyl acetate and water extracts. Sterols were present in extracts, obtained with hexane and methanol. Coumarins were present in water extract only.

Anthraquinone were present in methanol extract, Saponins not present in any extracts; in water extract the maximum number of phyto-constituents and the hexane and chloroform extract had the least number were noted, and extracts with the rest other solvents had medium levels of phyto-constituents (Table3)

Antibiotic sensitivity pattern

Antibiotic profile of each bacterial strain was determined using specified antibiotic discs (Table 4).

It was recorded that antibiotics (µg/disc), gentamicin 30 was resistant to six bacteria; norfloxacin 300 was resistant to three and sensitive to Three strains; nitrofurantoin 300 was resistant to one and sensitive to five strains; amikacin 30 was resistant to one and sensitive to five bacteria; cefotaxime 30 was resistant to six; imipenem 10 was resistant to five and sensitive to one; piperacillin/tazobactam 100/10 was resistant to five and sensitive to one bacterium; gatifloxacin 30 was resistant to Three and sensitive to Three isolates; ofloxacin 5 was resistant to Three and sensitive to Three strains; netilmicin 30 was resistant to four and sensitive to two isolates; amoxyclav 30 was recorded as resistant to four and sensitive to two bacteria.

Antibacterial Activities

Five pairs *P. integerrima* galls extracted with hexane, chloroform, ethyl acetate, methanol and water. Here screened for anti-bacterial activity against cited GP bacteria and three GN bacteria. Water extract only had the highest antibacterial activity against *E.coli*. (Table 5). Galls-extracts with water have shown significant antibacterial activity against all bacteria.

The maximum size of zone of inhibition had been recorded due to the water extract, as 15 mm against *K. pneumoniae and* MRSA which was 18 mm in the case of ciprofloxacin 30 µg/disc. Detailed information of antibacterial activities of

extracts and inhibition zone sizes were recorded (Table 5)

MIC and MBC Values

Particular galls-extracts obtained with different solvents that have shown significant antibacterial activity in the agar-well diffusion method were further used for the determination of MIC and MBC values with bacteria, in a 96-well micro-titre plate (Table 6). MIC values of all extracts ranged from 0.48 to 13.20 mg/mL, and MBC values ranged from 2.58to 30.00 mg/mL. The MIC value of the hexane extract ranged between 2.62 and 13.20 mg/mL, the MBC value ranged between 5.91 and 30.00 mg/mL; with chloroform extract, the MIC value ranged from 0.48 to 5.91 mg/mL, the MBC value ranged between 1.16 and 13.20 mg/mL; for ethyl acetate extract the MIC values ranged between 1.16 and 5.91 mg/mL, the MBC value was 2.58 to 13.20 mg/mL; the MIC value with the Methanol extract was 1.16-5.91 mg/mL and the MBC value ranged between 2.62and 13.20 mg/mL. The MIC value with the water extract was 2.62-5.91 mg/mL and the MBC value ranged between 5.91 and 13.30 mg/mL.

With *E. coli*, hexane, chloroform, ethyl acetate, methanol not detected MIC value water extract as5.91 mg/mL. With *K. pneumoniae*, the lowest MIC was with the methanolic extract as 1.16 mg/mL, and the highest MIC value was with the hexane, chloroform, ethyl acetate extract as 5.91 mg/mL. With *P. aeruginosa*, the lowest MIC was with Methanol extract at 1.16 mg/mL, the highest MIC value was with hexane, chloroform, water extract as 5.91 mg/mL. With *S. aureus*, the minimum MIC value was as 0.48 mg/mL and with the chloroform extract, and the maximum MIC value was with the hexane and water extract as 5.91 mg/mL. With MRSA, the minimum MIC value was with ethyl acetate extract 1.16 mg/mL, the maximum MIC value was as 5.91 mg/mL, due to methanol and water extracts. With VRSA, the minimum MIC value was with the chloroform extracts as 1.16 mg/mL, and the maximum MIC value was with the hexane extract as 13.20 mg/mL.

With *E. coli*, hexane, chloroform, ethyl acetate, methanol not detected MBC value water extract as 13.30 mg/mL.With *K. pneumoniae*, the minimum MBC value was 2.62 mg/mL in methanolic extract and the maximum value with hexane, chloroform and ethyl acetate extract was 13.20 mg/mL. With *P. aeruginosa*, the minimum MBC value with methanol extract was 2.62 mg/mL, the maximum MBC value with hexane, chloroform and water extract was 13.20 mg/mL. With *S. aureus*, the minimum MBC value with chloroform extract was 1.16 mg/mL, while the maximum value with hexane and water extract was 13.20 mg/mL. With MRSA, the minimum MBC value with ethyl acetate extract was 2.58 mg/mL, the maximum value with methanol and water extract was 13.20 mg/mL. With VRSA, the minimum MBC value with chloroform extract was 2.62 mg/mL, the maximum value with hexane extract was 30.00 mg/mL.

Identification and Characterization of Compound Isolated From Chloroform Fraction

Compound was obtained a white amorphous material, with a melting point range of 136-139°C.Dried crystals were reconstituted in chloroform and spotted on silica TLC plates and TLC was carried out using mobile phase of chloroform ethyl acetate (8:2 ratio) was detected on plates as the pink spot at the R*f*value of 0.3 on staining with 50% H_2SO_4. The compound was found to be UV active at 254nm. IR and NMR spectral analysis were done. The IR spectral analysis revealed a broad peak at 3426.3 cm-1 for OH group

The peaks at 2936, 2832, 2366 cm-1 indicate C=C group. The peaks at 1596.4 and 1032 cm-1 indicating the presence of C=O and C-O stretches, respectively (Yamaguchi, 1970). Elemental analysis and molecular weight determination indicated C_{29}-$H_{50}O$ as its molecular formula (Fig-2). In which IR peaks were obtained at 3426.89, 2924.52, 2855.1, 1738.51, 1057.31 cm-1. Further, evidence for its characterization came from its ^{1}H NMR spectrum which exhibited a broad triplet at δ 5.36 and a multiple at 3.50 corresponding to H-6 olefinic proton and H-3α proton respectively. Rest of protons appeared in the high field region in between 0.7-2.0 ppm.

The presence of flavonoids, tannins, alkaloids, sterols, coumarins and anthraquinonein galls-extracts was established; flavonoids were present in four-extract, whereas saponins absent in all extracts; alkaloids were present in three extracts, obtained with ethyl acetate, methanol and water, in this study. As reported, alkaloids, terpenoids, flavonoids, reducing sugar, soluble starch, and tannins were found in *P. integerrima* galls-extracts obtained with various fraction(Uddin G, *et al*., 2012);and alkaloids, tannins, flavonoids and sterols were recorded with *P. integerrima* stem bark chloroform and ethyl acetate fractions (Muhammad I, *et al*., 2011). Monoterpenes triterpenoids sterols, dihydromalvalic acid and flavonoids have been isolated from the different parts of *Pistacia* species (Ahmad NS, *et al*., 2010 and Ahmad NS, *et al*., 2008).

Pistaciai ntegerrima contain different phytochemicals including alkaloids, flavonoids, tannins, saponins, sterols and essential oils. Phytochemical analysis of *Pistacia integerrima* leaf was carried out and was found to contain carotenoids, triterpenoids, catechins and flavonoids (Ansari SH, *et al*., 1993 and Ansari SH,*et al*., 1994). *Pistacia integerrima* galls extracts presence alkaloids, terpenoids, flavonoids and tannins. Terpenoids and flavonoids in bark and leaves while roots of *Pistacia integerrima* were found to contain terpenoids and tannins. Fruits of *Pistacia integerrima* reported to possess tannins, essential oil, resin, pistacienoic acid, triterpene alcohol and triterpenoic acid (Prajapati ND, *et al*., 2006). Methanol extract and its ethyl acetate fraction of *Pistacia integerrima* were found to contain phenolics and flavonoids (Joshi UP, *et al*., 2009).

Pistacia integerrima represents one of those plants having broad-spectrum activities (Yamin B, *et al*., 2015). Antibacterial and antifungal activity of *Pistacia integrrima* stem were determined however, less antibacterial activity and more antifungal activity was observed (Aqil F, *et al*., 2003).

Crude and fractionated extracts of *Pistacia integerrima* stem were tested against different pathogenic bacteria. Crude extract showed maximum activity against *Pseudomonas picketti*. All fractions showed pronounced activity against *Salmonella setubal*and *Staphylococcus aureus,* however, maximum inhibition (19.66mm) were shown by aqueous fraction against *Bacillus subtilis* (Bibi Y, *et al*., 2011).Methanolic bark extract of *Pistacia integerrima* and solvent based fractions were subjected to anti-microbial activity and outstanding antibacterial activity was shown by ethyl acetate fraction against *Staphylococcus aureus*, however, extracts proved inactive for anti-fungal activity (Shafiqur Rahman, Ismail M, *et al*., 2011).

MRSA strains reported from Nepal were at 40.1% of the total bacterial isolates, and those strains were multiple resistant to trimethoprim/sulfamethoxazole, cephalexin, amikacin, ciprofloxacin and norfloxacin, in

addition to the usual penicillin derivatives, but all those were vancomycin sensitive (Tiwari HK,*et al*., 2009).

But the most effective way to prevent clinical crisis due to MRSA has been with daptomycin, nowadays (Holloway K. 2000 and Sorlozano A, *et al*., 2009). In Brazil, about 40% to 60% nosocomial infections in urinary and respiratory tracts, boils and surgical wound infections were by MRSA alone, and the presence of *mec*A gene with those was proved, probably because of such greater infection prevalence (Raja NS, 2007).In a study from Malaysia, it was reported that among 287 pathogens, 52% were GNs with *Proteus* sp. 25%, *P. aeruginosa* 25%, *K. pneumoniae* 15%, *E. coli* 9%, and the rest 45% were GP bacteria with *S. aureus* 40%, Group B *Streptococci* 25% and *Enterococcus* sp. 9%; antibiograms indicated the susceptibility to imipenem and amikacin in GN and vancomycin in GP bacteria(Kato T, *et al*., 1998).Among intracellular pathogens isolated, both *S. aureus* and *Staphylococcus epidermidis* were frequently present, the latter species being coagulase-negative *Staphylococcus*; and *S. aureus* strains were mostly MRSA. Indeed, *S. aureus* was not invasive intrinsically, but MRSA was reported as invasive through eye. Further, in a classical study from New York, it was reported that the colonization rate of MRSA was more in intravenous drug abusers (Berman DS, *et al*., 1987).

While analyzing the infection dynamics of pathogens, it was obvious that antibiotic sensitive pathogens have a limited capacity of virulence as the employed antibiotic controls them. At several levels, the host defence system also helps the control of pathogens when the later are in a smattering number.

Most often than not, an infection from a MDR bacterial strain leads to a disease, particularly when an emulating control-agent/antimicrobial is absent, *i.e.*, the employed antibiotic has been won over by it. Indeed, in the presence of a stress factor-an antibiotic, the bacterial cell undergoes intrinsic or acquired genetic changes via, conjugation/transformation, involving exchanges of resistance markers, exemplified with the *mar*-locus of *E. coli* (George AM, *et al*., 1983).if at least, the natural selection for the emergence of mutants is slow. Spontaneous mutation in bacteria occurs at the rate, 1 in 10^7 cells usually. Eventually, some drug-resistant mutant predominates with the replacement of all sensitive strains by the resistant strain, the later serving as if a doppelgänger. Since, the emergence of resistant mutants is a self-repetitive process in conditions ideal for pathogens, serial/continual resistant events to a gamut of diverse antibiotics land at the emergence of multidrug resistance in a bacterium, at least in an aged/immune-compromised body. Indeed, the horizontal transfer of genetic materials from one organism to another appears faster than mutational changes, a phenomenon popularly called as, evolution of quantum leaps, operates naturally(Groisman EA, 1996). It is because, genes for the drug-resistance mechanism are operative in antibiotic-producing cells, and those are transferred naturally to sensitive strains(Martinez JL, *et al* ., 2002), as an event of natural selection. Ultimately, antibiotic resistance remains as the clinical determinant of the pathogenesis. Slowly, the use of numbers of antibiotics for the control of infectious diseases in last decades have led to multiple resistances in one cell, the MDR strain of a species, paradigmatically with any of notorious pathogens.

Table.1 Isolation and Characterization of Pathogenic Clinical Isolates with Individual Colony Characteristics

Bacteria	Standard strain	Agar media	Colony morphology
E. coli	MTCC 443	Nutrient agar	Flat dry, irregular colonies
		MacConkey agar	LF, flat dry pink, irregular colonies
		EMB agar	Flat dry, irregular colonies, with metallic green colour
K. pneumoniae	MTCC 4031	MacConkey agar	LF, pink, mucoid colonies
P. aeruginosa	MTCC 1688	Nutrient agar	Large, irregular opaque colonies, with bluish green pigment
S. aureus, MRSA, VRSA	MTCC 7443	Blood agar	Medium to large, smooth, entire, slightly raised, creamy yellow, with green/β hemolytic colonies
		Nutrient agar	As in blood agar without hemolytic activity

MRSA: methicillin resistant S. aureus; VRSA: vancomycin resistant S. aureus; LF: lactose fermenting colonies; EMB: eosin methylene blue agar

Table.2 Summary of Results of Biochemical Tests of Six Pathogenic Bacteria

Bacterium	Catalase	Oxidase	Indole	MR	VP	Citrate	Urease	TSI	NT
E. coli	+ve	-ve	+ve	+ve	-ve	-ve	-ve	A/G	+ve
K. pneumoniae	+ve	-ve	-ve	-ve	+ve	+ve	+ve	A/GH_2S	+ve
P. aeruginosa	+ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	+ve
S. aureus, MRSA, VRSA	+ve	+ve	nd	nd	nd	nd	+ve	nd	nd

MR: methyl red; VP: Voges-Prausker; TSI: triple sugar iron; NT: nitrate reduction; A/G: acid and gas production; A/GH2S: acid-gas and hydrogen sulfide production; nd: not done; +ve: positive; -ve: negative

Table.3 Qualitative Phytochemical Analysis of Extracts of *P. integerrima* galls with different Solvents

Constituents	**He**	**Ch**	**Et**	**MeOH**	**H_2O**
Alkaloids	-	-	+	+	+
Coumarins	-	-	-	-	+
Flavonoids	+	+	+	-	+
Anthraquinone	-	-	-	+	-
Saponins	-	-	-	-	-
Tannins	-	+	+	-	+
Sterols	+	-	-	+	-

He: Hexane;Ch: Chloroform; Et: Ethyl acetate; Me: Methanol; H_2O: Water +: presence; -: absence of phytoconstituent

Table.4 Antibiogram of Clinically Isolated 6 Bacteria by the Disc-Diffusion Method with Antibiotics

Bacterium	**Set of antibiotics**												
	G	Nx	Nf	Ak	Ce	I	Pt	Gf	Of	Nt	Ac	Va	Ox
E. coli	R	R	S	S	R	R	R	S	R	S	R	-	-
K. pneumoniae	R	R	S	S	R	R	R	S	R	S	R	-	-
P. aeruginosa	R	R	R	S	R	S	S	S	R	R	R	-	-
MRSA	R	S	S	R	R	R	R	R	S	R	S	S	R
VRSA	R	S	S	S	R	R	R	R	S	R	R	R	R
S. aureus	R	S	S	S	R	R	R	R	S	R	S	S	S

Antibiotics (µg/disc): Ac: amoxyclav 30; Ak: amikacin 30; Ce: cefotaxime 30;G: gentamicin 30; Gf: gatifloxacin 30; I: imipenem 10;Nf: nitrofurantoin 300; Nt: netilmicin 30; Nx: norfloxacin 300; Of: ofloxacin 5; Ox: oxacillin 1; Pt: piperacillin/ tazobactam 100/10; Va: vancomycin 30. For *S. aureus*, oxacillin and vancomycin was used individually and lawns had no inhibition zone. R: resistance and S: sensitivity of a bacterium; -: antibiotic was not used. Data of the second repeated experiment are presented. All values are mean of duplicate readings

Table.5 Antimicrobial Activity (zone of inhibition in mm) of *P. integerrima* galls

Bacteria	**He**	**Ch**	**Et**	**MeOH**	**H_2O**	**Ciprofloxacin 30 µg/disc**
E. coli	-	-	-	-	14	16
K. pneumoniae	13	14	13	10	15	18
P. aeruginosa	12	13	13	11	14	17
S. aureus	14	13	12	14	14	19
MRSA	13	13	12	13	15	18
VRSA	12	13	13	12	13	18
He: Hexane; Ch: Chloroform; Et: ethyl acetate; MeOH: methanol; H_2O: Water -: absence of inhibition.						

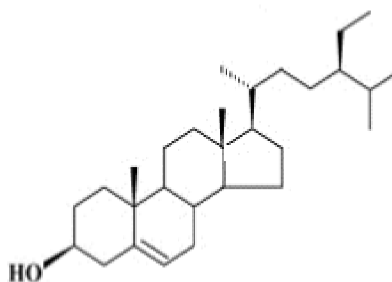
Table.6 MIC and MBC values of Galls-Extracts with Different Solvents against MDR Bacteria (mg/mL)

Bacteria	He		Ch		Et		MeOH		H_2O	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	ND	ND	ND	ND	ND	ND	ND	ND	5.91	13.30
K.pneumoniae	5.91	13.20	5.91	13.20	5.91	13.20	1.16	2.62	2.62	5.91
P.aeruginosa	5.91	13.20	5.91	13.20	2.62	5.91	1.16	2.62	5.91	13.20
S.aureus	5.91	13.20	0.48	1.16	2.62	5.91	2.62	5.91	5.91	13.20
MRSA	2.62	5.91	2.62	5.91	1.16	2.58	5.91	13.20	5.91	13.20
VRSA	13.20	30.00	1.16	2.62	5.91	13.20	5.91	13.20	5.91	13.20

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration;He: Hexane; Ch: Chloroform; Et: ethyl acetate; MeOH: methanol;H_2O: Water; ND: not done. Data of the second repeated experiment are presented

Fig.1 *Pistacia integerrima*: Galls

Fig.2 Compound Isolated from Galls of *Pistacia integerrima*. β-Sitosterol



As conjectured from retrospective follow-ups, it is clear that older antibiotics slowly became obsolete, by the resistant mechanism. The clinical concern is that antibiotic resistance was reported in several pathogenic bacteria for which, particular antibiotics were never applied. Is this the mechanism of the transformation of a harmless commensal to a perilous MDR pathogen in the present antibiotic era? Not surprisingly, drug resistant bacteria gain the capability of surviving and multiplying under stress conditions. The biological rule, any limiting condition for the majority would be an excellent opportunity for the minority. When in presence of a drug *in vivo*, all the drug sensitive strains are eliminated and the resistant strain survives, multiplies, and predominates, culminating in a disease. Drug resistant strains and their control by newer antibiotics are leitmotivs in the odyssey of the emergence of MDR and PDR strains of umpteen pathogens in the last 4-5 decades and more. MDR-MRSA is the intractable, ghoulish example rising to a great notoriety of being marked as the superbug of health domain, worldwide (Dubey D, *et al*., 2013).

Different isolation studies were conducted on galls extract of *Pistacia integerrima* and led to purification of hydroxyl-decanylarachidate, octadecan-9, 11-diol-7-one, β-sitosterol and pisticialanstenoic acid (Ahmed S, *et al*., 2010). Phenolic constituents characterised as 14′-phenoxytetradecany 3,5-dihydroxy benzoate (pistiphloro-glucinyl ester) 2,4′-phenoxy-*n*-butyl-1′-(3-oxy-5-hydroxy) benzoic acid (pistaciaphenyl ether) 3 and 3′-(1,3-dihydroxy-5-phenoxy-1′,5′-dimethox-benzene (pisticiphloro-glucinyl ether) (Ahmed S, *et al*., 2010). Furthermore ethyl gallate was isolated from galls of *Pistacia*

integerrima and suggested good for anti-inflammatory diseases (Mehla K, *et al*., 2011). Polyphenolic contents isolated from *Pistacia integerrima* leaves. β-sitosterol in addition to a new compound was isolated from cytotoxicchloroform and ethyl acetate fraction of methanol stem extract (Bibi Y, *et al*., 2011). Pistagremic acid was isolated from whole plant extract of *Pistacia integerrima* and exhibited significant leishmanicidal activity (IC (50): 6.71±0.09μM) against Leishmania major (DESTO) promastigotes in comparison to standard compound amphotericin (Uddin G, *et al*., 2011b).As reported molecular formula of β-sitosterol (Karan SK, *et al*., 2012);and reported characterized the presence of β-sitosterol by ^{1}H NMR at δ 5.39 (1H, m H6) and 3.51 (1H, m H3)(Parveen S, *et al*., 2011).Also reported the presence and characterization of β-sitosterol in *Withania somnifera* L. in which broad peak was obtained in IR spectra at 37000-36000 cm-1 (OH group), 2790 (CH stretch), 2995 to 2995 cm-1 show resonance with the C=O group while NMR spectrum showed the existence of proton at 7.4 ppm and multiple at 3.4 ppm (Trivedi PC, *et al*., 2011).

As reported, β-sitosterol were found in TLC Presence of β-sitosterol has been reported in various plants, such as leaves of *Ocimum sanctum* (Rahman SMM, *et al*., 2009). Rhizomes of the *Stylochiton lancifolius* (Pateh U, *et al*., 2009). fruits of *Coryluscolurna* Linn (Akhtar P, *et al*., 2010); and *Solanum xanthocarpum* (Khanam S, *et al*., 2012).as well as in the tissue cultures of *Adhatodavasica* & *Ageratum conyzoides*(Sarin R, *et al*., 2011); and cell suspension culture of *Chrysanthemum coronarium* L (Kim HC, *et al*., 2005); and galls of the *P. integerrima* (Ahmad S, *et al*., 2010).

β-sitosterol is a natural micro-nutrient which is found in the cells and membranes of all oil producing plants, fruit, vegetables, grains, seeds and trees. It has been proven to be a safe, natural and effective nutritional supplement and has shown amazing potential benefits in many diverse applications. Earlier experimental studies have shown its effectiveness as an anti-diabetic, antioxidant, anti-cancer, anti-ulcer, anti-inflammatory, antipyretic and anti-stress agent. This natural micro-nutrient is also an effective immune booster and used in the treatment of prostate enlargement and HIV. Our study contributes to establish β-sitosterol as potent antimicrobial agent at lower concentration against a wide range of bacteria. However, still more scientific evaluation and clinical trials are required to establish its therapeutic efficacy.

In conclusion from the recorded data, it could be taken that *P. integerrima* galls-extract could be used in treating infectious diseases, caused by the range of tested bacteria, as complementary and alternate medicine, since crude phyto-extracts of the plant could not be breached by MDR pathogenic bacteria. Apothecary would benefit from these findings of the plant for drugs of finesse, *i.e.*, non-microbial antimicrobials in the crusade against MDR pathogens.

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